Proteinase 3

A Distinct Human Polymorphonuclear Leukocyte Proteinase that Produces Emphysema in Hamsters

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Abstract

Studies were designed to explore the possibility that human polymorphonuclear leukocyte granule constituents in addition to elastase (HLE) had the potential to cause emphysema. A two-step purification of three serine proteinases was developed. Granule extract proteins were initially separated by dye-ligand affinity chromatography. Fractions eluted were divided into four pools. Hamsters were given a single intratracheal instillation of saline ± 0.1 mg protein of each pool. While pool 2 contained HLE and cathepsin G, the most dramatic bullous emphysema developed in animals treated with pool 4. The esterase from pool 4, designated proteinase 3 (PR-3) was purified, characterized in vitro, and tested for its ability to cause emphysema. PR-3 is a neutral serine proteinase with isoenzyme forms. Its ability to degrade elastin at pH 6.5 is slightly greater than that of HLE, but it is less active than HLE at pH 7.4 or 8.9. PR-3 has weak activity against azocasein. Its ability to degrade hemoglobin is intermediate to that of HLE and cathepsin G at pH 7.4. PR-3 has no activity against chromogenic substrates specific for HLE or cathepsin G. Its *pI* is substantially less than HLE or cathepsin G. It is also immunologically distinct from HLE. It induces emphysema in hamsters commensurate with that of HLE. We conclude that PR-3 may be important in the pathogenesis of human emphysema.

Introduction

Proteinases released into the extracellular space by polymorphonuclear leukocytes (PMN) have the potential to produce tissue injury. Human leukocyte elastase (HLE)¹ has attracted particular attention in this regard because of its putative role in the pathogenesis of pulmonary emphysema (1, 2). Thus far, HLE has been the only proteinase purified from a cell with access to the lungs that has been shown to directly induce emphysema in animal models.

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1. *Abbreviations used in this paper:* BAPN, β -aminopropionitrile; CPB, citrate phosphate buffer; DFP, diisopropyl fluorophosphate; HLE, human leukocyte elastase; ISA, internal alveolar surface area; L_m , linear intercept; OA-P1, 2, 3, or 4, Orange A Pool 1, 2, 3, 4; PR-3, proteinase 3.

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However, to consider only HLE-mediated injury in examining the pathogenesis of emphysema in the human is too narrow a focus. A direct relationship of HLE to the human disease remains to be demonstrated. Recent studies suggest that the increased risk of emphysema associated with cigarette smoking may be due partially to the effect of phagocyte or smoke-derived oxidants in the lung (3, 4). Another variable immediately tangent to the HLE pathogenesis concept is that other PMN or alveolar macrophage proteinases may participate in matrix destruction. Cathepsin G and an AM cysteine proteinase(s) both have elastolytic capabilities in vitro (5, 6). Moreover, cathepsin G has been reported to act synergistically with HLE in the solubilization of elastin in vitro (7), although results have been discrepant (8, 9). Studies to date that have failed to demonstrate that these proteinases can produce emphysema (8) do not exclude their participation in the induction of the disease. The in vivo action of PMN proteinases on the connective tissue substrate of the lung should be further studied individually and synergism of the enzymes should be explored.

In this study we explored the possibility that a human PMN proteinase, distinct from HLE and cathepsin G, had the potential to cause emphysema. We initially demonstrated the emphysema producing capacities of a granule extract separated from HLE and cathepsin G by dye ligand affinity chromatography. We purified this proteinase and identified it as proteinase 3 (PR-3) a neutral serine proteinase briefly described by Baggiolini, which acts preferentially on α -naphthyl acetate (10). We further characterized the physical and functional properties of PR-3 and tested its ability to cause emphysema in hamsters. The results suggest that PR-3 is a distinct PMN proteinase that can degrade elastin in vitro and can produce emphysema commensurate to that produced by HLE.

Methods

Preparation of leukocyte granule proteins

Isolation of PMN. Leukocyte concentrates were prepared from pooled, normal human blood and were subjected to red cell sedimentation using 6.0% dextran in physiologic saline. The supernatant was removed and centrifuged at 400 g for 10 min. To lyse contaminating red blood cells, pellets were resuspended in plasma and 0.87% NH_4Cl for 4 min, recentrifuged, and then subjected to hypotonic shock (11, 12). After centrifugation at 400 g for 10 min, all leukocytes were washed and resuspended in HBSS, counted, and a sample sedimented onto a glass slide, then stained with Wright-Giemsa for determination of differential cell populations. 85-95% of the isolated cells were PMN. In selected experiments the cells were purified by Ficoll-Hypaque gradient centrifugation before red cell lysis, washed and resuspended in HBSS (13). These preparations contained > 97% PMN and < 3% eosinophils.

Isolation of cytoplasmic granules and extraction of granule proteins. Approximately 5×10^8 PMN were suspended in 2 ml of 0.34 M sucrose and homogenized at 4°C with a motor-driven homogenizer

with Teflon pestle for two intervals of 3 min each. The homogenate was centrifuged at 200 g/10 min/4°C, the supernatant removed, and centrifuged at 8,700 g/20 min/4°C. The pellet contained the mixed granule fraction and was extracted immediately or stored at -70°C. Granule pellets from 3×10^{10} PMN were suspended in 12 ml cold 0.01 N HCl and sonicated at 45 kHz for three 30-s intervals. The extract was centrifuged at 105,000 g/1 h/4°C, the supernatant fluid was decanted, and used immediately for purification of proteinases by column chromatography.

Chromatographic separation of proteinases from granule extracts

Dye ligand affinity chromatography. Proteins in the granule extract from 3×10^{10} PMN were first separated by dye-ligand affinity with a 28×1.8 cm Matrex Gel Orange A (Amicon Corp., Danvers, MA) column (12). Approximately 150 mg protein was applied to the column and allowed to bind at room temperature for 1 h. The column was washed with 100 ml of 0.08 M citrate phosphate buffer (CPB), pH 5.6, followed by 150 ml of 0.1 M NaCl in CPB. Bound proteins were eluted with a gradient from 0.1 to 1.6 M NaCl in CPB. Samples of alternate 5-ml fractions were removed for determination of protein using the Hartree modification of the Lowry method (14). NaCl content of the fractions was determined by measuring their conductivity using a conductivity meter (CD-M3; Radiometer, Copenhagen, Denmark). Esterase activity was measured using α -naphthyl acetate as described below (15). A representative profile of the separation of granule proteins by Orange A column chromatography is shown in Fig. 1. The eluted fractions were combined into four pools designated Orange A Pool 1, 2, 3, or 4 (OA-P1, 2, 3, or 4). OA-P2, which eluted between 0.30 and 0.74 M NaCl, and OA-P4, which eluted between 0.90 and 1.1 M NaCl, contained the esterase activity and were further studied. The major esterase peak in OA-P2 (fractions 25–55) was identified as HLE by its ability to cleave the elastase specific chromogenic substrate, succinyl-(alanyl)₃-p-nitroanilide (Suc-Ala₃-pNA). The minor peak in OA-P2 (fractions 56–65) was identified as cathepsin G by its ability to cleave the chymotrypsin specific substrate, succinyl(alanyl)₂-prolyl-phenylalanine-p-nitroanilide (Suc-Ala₂-Pro-Phe-pNA). The esterase peak in OA-P4 (fractions 81–100) did not react with either substrate.

Cation exchange chromatography. Pools OA-P2 and OA-P4 were further purified using a Bio-Rex 70 cation exchanger (Bio-Rad Laboratories, Richmond, CA). The cation exchange resin was converted with 0.5 M NaOH and washed thoroughly with 0.08 M CPB. A 7.0×1.0 -cm column was poured and was equilibrated to 0.05 M NaCl in CPB, pH 7. Pool OA-P2 or OA-P4 was diluted to 0.05 M NaCl, applied to the column and washed with 0.05 M NaCl in CPB (pH 7.0). Bound proteins were eluted with a gradient from 0.05 to 1.0 M NaCl in CPB, pH 7.0. Protein, conductivity, and nonspecific esterase activity were determined on samples from alternate 5-ml fractions. After cation exchange chromatography, esterases were concentrated in an ultrafiltration unit (Amicon Corp.), adjusted to physiologic saline concentration, and filter sterilized. Final protein concentration was measured (14) using BSA as a standard.

Overall, the two step purification of HLE and cathepsin G resulted in the recovery of 34.2 and 53.2%, respectively, of these proteinases. This represents an 8.7-fold purification of HLE and a 23.5-fold purification of cathepsin G. Percent recovery of PR-3 cannot be determined because a specific substrate is not yet available. However, the cation exchanger increased the specific activity of the esterase in OA-P4 from 96 nm esterase activity/mg protein to 280 nm esterase activity/mg protein.

Induction of emphysema

Female Syrian golden hamsters (Harlan Industries, Indianapolis, IN) weighing 90–110 g were used. In the initial studies, diets were supplemented with 0.5% (by weight) β -aminopropionitrile (BAPN) starting 1 wk before intratracheal instillation and thereafter until killed. BAPN is a lathyrogen that inhibits lysyl oxidase and acts to enhance the

severity of emphysema in the presence of elastin degradation (16). Pentobarbital anesthetized hamsters were given a single intratracheal injection of 0.5 ml physiologic saline or 0.5 ml physiologic saline containing 0.1 mg of OA-P1, 2, 3, or 4. 30 wk after treatment, anesthetized animals were killed by exsanguination, the trachea and lungs were excised en bloc, inflated, and fixed in 10% formalin at constant pressure (25 cm H₂O) for 24 h. Lung volumes were determined by water displacement. A midsagittal section cut from each lung was processed for histologic studies. The extent of emphysema was determined on coded and randomized sections by measuring the mean linear intercept (L_m) in 20 randomly selected fields from each lung (17). Internal alveolar surface area (ISA) was calculated from the lung volumes and intercepts, and corrected to a constant lung volume of 4 ml (ISA₄) (18). Each category of measurement was subjected to an analysis of variance and the differences between the group means evaluated by Student-Newman-Keul's test.

In subsequent experiments, hamsters fed a normal diet (non-BAPN supplemented) were anesthetized and given a single intratracheal injection of 0.5 ml saline or 0.5 ml saline containing 0.1 mg of OA-P4, PR-3 that had been purified from OA-P4, or HLE, which had been purified from OA-P2. The animals were killed 10 wk later, the lung volumes were determined, and the degree of emphysema was assessed morphometrically.

Characterization of PR-3

Electrophoresis. PAGE was done as described by Laemmli (19). The proteins were stained with Coomassie Blue R-250 (0.125% in 45% methanol, 10% acetic acid) overnight at room temperature and destained with SDS-Coomassie destain (30% methanol, 8% acetic acid). Discontinuous nondenaturing gel electrophoresis was done by the method of Thomas and Hodes (20). The proteins were stained for esterase with 50 mg of α -naphthyl acetate dissolved in 20 ml of dimethylformamide and 80 ml of 0.2 M potassium phosphate buffer, pH 7.0, to which was added 0.2 g Fast Garnet GBC salt. Gels were immersed in stain for 2 h at 37°C and then transferred to 7.0% acetic acid.

Elastin degradation. Bovine ligament elastin was prepared by the method of Starcher and Galione (21) and assessed for purity by amino acid analysis. Elastin degradation was assayed by determining the ability of the proteinase to solubilize elastin radiolabeled with [³H]NaBH₄ over a pH range from 6.5 to 8.9 using the procedure of Stone et al. (22). The tritiated powdered elastin was washed and resuspended in 0.2 M sodium phosphate buffer, pH 6.5 or 7.4, or sodium bicarbonate buffer, pH 8.9. The proteinase in physiologic saline was added to a 5-mg aliquot of ³H-elastin and the reaction mixture was incubated at 37°C for 6 h. After incubation, the contents of each tube were filtered through a medium porosity filter paper to remove the insoluble elastin. The rate of degradation was determined by measuring the radioactivity in the filtrate.

Two confirmatory assays of elastin degradation were used: radial diffusion in agarose gels containing elastin, and measurement of desmosine content of supernatants of digests from unlabeled insoluble elastin. Radial diffusion in agarose gels containing elastin employed a modification of the method of Schumacher and Schill (23). Agarose was dissolved at 10 mg/ml in 0.2 M Tris buffer, pH 8.9, and boiled for 20 min. Once the mixture had cooled to 50°C, finely pulverized elastin (mesh size < 400) was added to the agarose and suspended by 10 s of vortexing. Sodium azide (0.05%) was added to the mixture to prevent bacterial growth. Aliquots were poured onto 3.5-cm diam tissue culture plates. Plates were stored in moist chambers at 4°C before use. For assay of elastase activity, 3-mm diam wells were cut into the gel. Proteinase samples (7.5 μ l) were placed into the gel and incubated at 37°C in a moist chamber. Zones of lysis were examined at 8-h intervals.

Desmosine content of supernatants of a 1-ml reaction mixture containing the proteinase in PBS, pH 7.4, and 5 mg of unlabeled insoluble elastin was determined using an ELISA method, which is sensitive to a level of 2.5 pmol (24). An inhibition assay was used in which the sample containing desmosine was preincubated overnight with antidesmosine antibody (1:1,000–1:4,000 dilution). After prein-

cubation, the sample plus antibody were added to 96 well polyvinyl plates coated with desmosine conjugated to gelatin, incubated for 30 min at 37°C, and washed. Second antibody (an alkaline phosphatase labeled anti-rabbit IgG) was incubated in wells for 1 h and the unbound antibody removed by repetitive washing. The alkaline phosphatase activity was determined by addition of *p*-nitrophenyl phosphate.

Azocasein degradation. Cleavage of azocasein was determined by the method of Starkey and Barrett (25). 20 µg of proteinase were added to 0.5 ml of an azocasein solution (6% in 0.1 M Tris, pH 7.0) and the final volume brought to 1.0 ml by addition of 0.1 M Tris, pH 7.0. Reaction mixtures were incubated at 37°C for 90 min and then precipitated with 3.0 ml of cold 5.0% TCA. The precipitate was removed by centrifugation at 200 *g* for 10 min. Supernatant was removed and measured at A_{410nm}.

Hemoglobin degradation. Hemoglobin degradation was determined by a modification of the method originally described by Anson (26). The proteinase was incubated with 1% nondenatured hemoglobin in 0.01 M phosphate buffer, pH 7.2, at 37°C for 20 h. Undegraded hemoglobin was precipitated with 5% TCA and pelleted by centrifugation at 10,000 *g* for 10 min. The absorbance of the supernatant was measured at A_{280nm}.

Cleavage of synthetic peptides. Synthetic chromogenic peptide substrates were used to characterize PR-3. The substrates included Suc-Ala₃-pNA or Suc-Ala₂-Val-pNA, substrates for elastase, and Suc-Ala₂-Pro-Phe-pNA, a substrate for chymotrypsin-like enzymes. The method used for these substrates was that described by Barrett (27), with some modification. Briefly, 50 µl of substrate (10 mg/1.1 ml DMSO) was added to 0.1 M Tris buffer, pH 7.0. The reaction was started by the addition of the proteinase for a final volume of 3.0 ml. Cleavage was measured by the increase of OD at A_{410nm}.

Trypsin-like activity of PR-3 was assessed by determining the role of hydrolysis of tosyl-Arg-methyl ester based on the method of Hummel (28). Briefly, 300 µl of substrate (37.9 mg/10.0 ml H₂O) was added to 2.4 ml of 0.05 M Tris buffer containing 10 mM CaCl₂ (pH 8.1). The reaction was started by the addition of the proteinase for a final volume of 3.0 ml. Hydrolysis was measured by an increase in OD at A_{247nm}. A blank without enzyme was included to monitor for spontaneous hydrolysis of the substrate.

Characterization of the chemical nature of this essential catalytic group of PR-3. The α -naphthyl acetate esterolytic activity of PR-3 in the presence or absence of inhibitors with relative group specificity was tested to determine the catalytic classification of PR-3. PMSF and diisopropyl fluorophosphate (DFP) were used as group-specific inhibitors of serine proteinases. 1,10 phenanthroline was used as a metallo-proteinase inhibitor. *N*-ethylmaleimide and 4-chloromercuribenzoate were used as representatives of thiol-proteinase inhibitors and pepstatin was used as a carboxyl proteinase inhibitor.

Cleavage of α -naphthyl acetate was measured using a modification of the method of Barrett (15). Briefly, each 0.5-ml sample was added to 1.5 ml of 0.1 M Tris/HCl, pH 7.0, followed by 20 µl of a 4-mg/ml stock solution of α -naphthyl acetate in DMSO. The samples were incubated for 1 h at 37°C. After incubation, 2.0 ml of coupling reagent was added to each sample and the color allowed to develop for 5 min before reading spectrophotometrically at A₅₂₀. Coupling reagent consisted of 30 mg Fast Garnet GBC salt in 50 ml of 2.0% Brij 35 solution plus 50 ml of 1.0 M potassium phosphate buffer, pH 6.0, with 0.015 M EDTA. The amount of substrate cleaved was determined by comparison to a standard curve made with β -naphthol. The percent activity was calculated by determining the difference in the proteinase-induced naphthol release in the presence or absence of inhibitor. Selected inhibitors were tested for their ability to block the elastinolytic activity of PR-3 using the [³H]elastin assay described previously in Methods.

Isoelectric focusing. Isoelectric focusing was done in ultrathin gels using LKB ampholine PAG plates, pH range 3.5–9.5, and the Multiphor electrophoresis unit (LKB-Produkter AB, Bromma, Sweden). The gels were run at 10°C at 15 mA constant current for 1.5 h. The gels were fixed, stained, and destained by the same procedure used for SDS-PAGE.

Immunoprecipitation studies. Proteinase samples were radiolabeled with ¹²⁵I as previously described (29). Briefly, 20 µl of sample, 100 µl of 0.5 M sodium phosphate, pH 7.5, and 50 µl of 10 mM sodium phosphate, pH 7.5, containing 100 µCi of ¹²⁵I-Na, were mixed in a siliconized 12 × 75-mm glass test tube. 50 µl of water containing 1 mg/ml of chloramine *T* was added and the mixture was incubated 1 min at 23°C. The reaction was terminated by adding 25 µl of water containing 250 µg of Na₂S₂O₃ and 50 µl of 0.5 M NaI. The resulting sample was desalted on Sephadex G-25 and either used for immunoprecipitation studies as described below or was analyzed by SDS-PAGE.

Radiolabeled proteins were immunoprecipitated as previously described with minor modifications (29). Briefly, ¹²⁵I-labeled protein was mixed with antibody in a buffer containing 20 mM Tris-HCl, pH 8.2, 100 mM NaCl, 0.5% NP-40, 1 mM EDTA, 2 mM PMSF, and 0.125 mg/ml gelatin. The total volume was 0.25 ml in 10 × 75 mm siliconized glass tubes. Rabbit anti-HLE prepared by Dr. Philip J. Stone (Boston University Medical School, Boston, MA), was a gift of Dr. Stephen McGowan, University of Minnesota Medical School. After incubating the suspension overnight at 0°C, 50 µl of 10% *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring Corp., La Jolla, CA) was then added. After 15 min at 0°C, the mixture was washed twice by adding 2 ml of buffer containing 20 mM Tris-HCl, pH 8.2, 1 mM EDTA, 100 mM NaCl, 0.5% NP-40, 2.5 M KCl, and 0.25 mg/ml gelatin, and centrifuging at 2,000 *g* for 20 min at 4°C. The pellet was then suspended in 1 ml of buffer containing 20 mM Tris-HCl, pH 8.2, transferred to an Eppendorf tube, and centrifuged for 5 min in an Eppendorf centrifuge. The precipitate was suspended in sample buffer and analyzed by SDS-PAGE. Gel slabs were stained, dried, and examined by autoradiography.

Results

The possibility that PMN granule components in addition to those containing HLE had the potential to cause emphysema was initially explored with the pooled fractions from the initial Orange A purification step (Fig. 1). Each pool was concentrated, and adjusted to physiologic saline concentration and filter sterilized prior to intratracheal instillation. The lungs were examined thirty weeks later. The most striking abnormalities were seen in animals receiving OA-P4. The lungs from four of five animals receiving OA-P4 contained multiple large (up to 1 cm diam), thin walled-subpleural bullae (Fig. 2 *a*). Small bullae were present in the lungs of one of five animals receiving OA-P2. Light microscopy of lungs fixed in inflation

Figure 1. Profile of chromatographic separation of PMN granule proteins by dye-ligand affinity with Matrex Gel Orange A. The eluted fractions were combined into four pools as indicated.

Figure 2. (a) Air-filled lung from a hamster that had received OA-P4 30 wk before. Multiple large subpleural bullae were prominent in this treatment group. (b) Histologic section of an inflation fixed lung of animal in the same treatment group showing emphysematous changes (hematoxylin-eosin stain; original magnification 180). (c) Normal hamster lung processed similar to that in b.

at constant pressure (25 cm H₂O) revealed widespread airspace enlargement and alveolar septal disruption in all lungs of OA-P4 animals (Fig. 2 b). Lesser degrees of airspace enlargement were observed in the lungs of animals receiving OA-P2. No abnormalities were noted in saline controls (Fig. 2 c) or animals receiving OA-P1 or OA-P3. To quantify the extent of airspace enlargement, the L_m was measured on each lung section (Fig. 3). The average L_m in OA-P4 animals ($124 \pm 9 \mu\text{m}$, mean $\pm$ 1 SEM) was significantly increased over that of controls (49.7 ± 4 , $P < 0.01$). The mean L_m in OA-P2 animals (90 ± 5) was greater ($P < 0.01$) than controls, but less ($P < 0.05$) than OA-P4. The lungs from animals receiving OA-P1 or OA-P3 did not have airspace enlargement ($L_m = 51.9 \pm 6$ or 58 ± 3 , respectively). We concluded that a PMN granule product in OA-P4 had the ability to cause emphysema in BAPN-treated hamsters that was of greater severity than that produced by the pool containing HLE, OA-P2.

These initial results encouraged us to undertake purification and characterization of the esterase in OA-P4. Cation exchange chromatography resulted in a single peak of esterase

activity that eluted at 0.11 M NaCl in CPB pH 7.0, designated PR-3 (Fig. 4). This peak was present in the PMN isolated from Ficoll-Hypaque centrifugation, but not the mononuclear cells, establishing that PMN were the source. SDS-PAGE revealed three bands (Fig. 5) suggesting isoenzyme forms were present. The molecular weight of the major band of PR-3 was $\sim 26,800$ D with the minor bands having slightly larger molecular mass.

In light of the initial studies suggesting that PR-3 could induce emphysema, its elastinolytic activity was thoroughly evaluated. Assays employing ³H-elastin (Table I) demonstrated that PR-3 hydrolyzed more elastin than HLE (wt/wt) at pH 6.5, that its activity at pH 7.4 was $\sim 50\%$ that of HLE and that it was much less active than HLE at pH 8.9. Assays of desmosine release (Table I) confirmed the activity of PR-3 against elastin at pH 7.4 was $\sim 50\%$ that of HLE. Finally, PR-3 digested elastin in agar with $\sim 50\%$ the activity of HLE at pH 8.9 (Fig. 6). Under all assay conditions used, PR-3 was much more active against elastin than was cathepsin G.

Some additional characteristics of PR-3 compared to those

Figure 2 (Continued)

Figure 3. Mean linear intercepts (L_m) of lungs from animals receiving OA-P1, 2, 3, or 4 or saline controls. Values are expressed as the mean $\pm$ SEM. L_m of lungs from animals receiving OA-P2 or OA-P4 are significantly different from saline control ($P < 0.01$).

of HLE and cathepsin G, which were also purified by cation exchange chromatography, are summarized in Table II. PR-3 was weakly active against azocasein, but had activity against hemoglobin intermediate to that of HLE and cathepsin G. PR-3 did not hydrolyze the substrates specific for HLE (Suc-Ala₃-pNA, or Suc-Ala₂-Val-pNA), for cathepsin G (Suc-Ala₂-Pro-Phe-pNA) or for trypsin (tosyl-Arg-methyl ester). The pI of the major band of PR-3 was 9.1, indicating that it was less cationic than either HLE or cathepsin G, which have pIs of 11 and greater than 11, respectively, by similar methods of analysis (30). Similarly, the mobility of the three bands of PR-3 in nondenaturing gel was distinctly slower than HLE or cathepsin G (Fig. 7, and Table II). The pH optimum for the esterase activity with α -naphthyl acetate of PR-3 was 7.0, indicating that it is a neutral proteinase.

Assignment of PR-3 to a catalytic class utilized inhibitors with relative class specificity (Table III). The esterase activity of PR-3 was markedly inhibited by PMSF and DFP, but not by 1,10 phenanthroline, *N*-ethylmaleimide, 4-chloromercuribenzoate or pepstatin. PMSF also completely inhibited PR-3's elastinolytic activity at pH 7.4. The results indicate that PR-3 is a serine proteinase. Neither the HLE specific inhibitor methoxysuccinyl-alanyl-alanyl-prolyl-valine chloromethyl ketone nor the cathepsin G inhibitor Z-glycyl-leucyl-phenylalanine chloromethyl ketone were effective inhibitors of PR-3's elastinolytic activity (data not shown).

To further document the uniqueness of PR-3, purified human neutrophil elastase and PR-3 were radiolabeled with ¹²⁵I using chloramine T, and immunoprecipitated with polyclonal rabbit anti-HLE (Fig. 8). Purified elastase was readily immunoprecipitated by rabbit anti-HLE, but not by normal

Figure 4. Profile of separation of OA-P4 proteins by Bio-Rex 70 cation exchange chromatography. The fractions of the major protein peak associated with the esterase peak comprise PR-3.

Figure 5. SDS-PAGE of PR-3. Lane A was run with crude granule extract. Lane B and lane C were applied with 25 μ l of PR-3 from two separate purification procedures (245 and 418 μ g/ml, respectively). Lane D was run with 10 μ l of SDS-standards (2 mg/ml) comprised of 66,000 D (66 kD), egg albumin (45 kD), glyceraldehyde-3-phosphate (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), trypsin inhibitor (20 kD), and α -lactalbumin (14 kD). The gel was visualized with Coomassie Blue stain. Note the relative purity of PR-3 in lanes B and C.

rabbit serum. In contrast, purified PR-3 was not immunoprecipitated by either rabbit anti-HLE or normal rabbit serum.

To strengthen our studies on the emphysema producing

Table I. Degradation of Elastin by Purified PMN Proteinase

	³ H-Elastin			Desmosine released
	pH 6.5	pH 7.4	pH 8.9	pH 7.4
	cpm released/5 mg ³ H-elastin/6 h			pmol/5 mg elastin/18 h
PR-3	1,710 $\pm$ 50*	2,250 $\pm$ 30	2,100 $\pm$ 120	2,750
HLE	1,190 $\pm$ 240	4,470 $\pm$ 300	18,770 $\pm$ 640	5,400
Cathepsin G	ND	408*	890 $\pm$ 50	540

* All values are corrected for background activity by subtracting radioactivity in buffer controls. Background activity was always $< 10\%$ of most active fraction. Values are expressed as mean $\pm$ SE.

† Mean of duplicate determinations.

Figure 6. Elastinolysis by PR-3 or HLE in elastin agar gels. Plates were incubated at 37°C for 16 h. (a) PR-3. (i) 1.8 μ g of purified enzyme was added to well. (ii) 0.9 μ g of purified enzyme was added; (b) HLE. (i) 1.8 μ g of purified enzyme was added. (ii) 0.9 μ g of purified enzyme was added.

potential of PR-3, we compared the ability of OA-P4, purified PR-3 and HLE to induce emphysema in hamsters fed a normal diet (non-BAPN supplemented). OA-P4 or the purified

enzymes were concentrated, adjusted to physiologic saline concentrations, and administered to anesthetized hamsters by intratracheal instillation of 0.1 mg protein in 0.5 ml saline.

Table II. Characteristics of PR-3 Compared to HLE and Cathepsin G

Substrate specificity*	PR-3	HLE	Cathepsin G
%			
Azocasein	14±5	58±8	100
Hemoglobin	84±8	100	54±10
Synthetic peptides			
Suc-Ala ₃ -pNA	0	100	0
Suc-Ala ₂ -Val-pNA	0	100	0
Suc-Ala ₂ -Pro-Phe-pNA	0	0	100
Tosyl-Arg-Methyl ester	0	ND	ND
pI	9.1	>9.5	>9.5
R _f	0.29	0.74	1.14

* Values for substrate specificity are expressed as a mean percentage±standard error of the enzyme most active with each substrate (100%).

R_f Electrophoretic mobility of the major band relative to cytochrome *c* (R_f = 1.0) after nondenaturing polyacrylamide gel electrophoresis. Separation of the proteins by this method is dependent on both the molecular weight and the isoelectric point.

The animals were killed 10 wk later. The results of the morphometric analysis are presented in Table IV. OA-P4 caused emphysema even in the absence of a lathyrogen, as evidenced by the large increase in *L_m* (*P* < 0.05) and the decrease in ISA compared with the saline controls. The *L_m* and ISA values after administration of PR-3 also indicated emphysema, commensurate with that of HLE. In short, it is clear that PR-3 is a potent inducer of emphysema.

Discussion

The major finding of this investigation is that a distinct neutral serine proteinase in human PMN, PR-3, has the ability to cause emphysema in hamsters, which is of equal or greater

Figure 7. Comparison of migration of purified PMN proteinases in nondenaturing polyacrylamide gel electrophoresis stained for esterase activity with α -naphthyl acetate. Lane A: PR-3, 150 μ g protein, lane B: HLE, 150 μ g protein, lane C: cathepsin G, 150 μ g protein. Migration of proteins by this method is dependent on both the molecular weight and the isoelectric point.

Table III. The Effects of Potential Inhibitors on Proteinase 3 Activity

Inhibitor	Final concentration	Esterolytic activity
	mM	%
None	—	100%
PMSF	1 mM	0
DFP	1 mM	9
1,10-Phenanthroline	1 mM	103
N-ethylmaleimide	1 mM	100
4-Chloromercuribenzoate	1 mM	97
Pepstatin	1 μ g/ml	97

* Enzymatic activity is expressed as a percentage of the proteinase-induced naphthol release in the absence of inhibitors. Inhibitors were preincubated with PR-3 for 1 h at 22°C before determining the α -naphthyl acetate esterolytic activity.

severity than that produced by a similar amount of HLE. To our knowledge, PR-3 is only the second enzyme purified from human phagocytes that has been demonstrated to cause experimental emphysema. To put this work into perspective and to better understand the possible implications, it will be useful to review other observations that have been made to date.

PR-3 was described by Baggiolini et al. in 1978 as a result exclusively of observations made after nondenaturing polyacrylamide gel electrophoresis of extract of azurophilic granules from PMN (11). We have concluded that the esterase purified from OA-P4 is the same enzyme. In their report, Baggiolini et al. found the electrophoretic mobility of PR-3 in nondenaturing gels was distinctly slower than that of HLE or cathepsin G. In addition to being a neutral esterase (it was stained with naphthol AS-D acetate and α -naphthyl acetate), and having a serine active site (staining was inhibitable by

Figure 8. Immunoprecipitation and PAGE of 125 I-labeled, purified HLE and PR-3. Aliquots of purified proteinases were labeled with 125 I using chloramine T, immunoprecipitated, and analyzed by SDS-PAGE and autoradiography as described in the text. Approximately 2×10^5 cpm of 125 I-labeled protein was used for each immunoprecipitation reaction. Lane A: purified

125 I-labeled HLE, lanes B and C: immunoprecipitates of 125 I-labeled HLE, lane D: purified 125 I-labeled PR-3, lanes E and F: immunoprecipitates of 125 I-labeled PR-3. Immunoprecipitating antibodies: polyclonal antineutrophil elastase (lanes B and E), normal rabbit serum (lanes C and F). Proteins used as molecular weight standards were: *Escherichia coli* beta galactosidase, 116,000; BSA, 66,000; ovalbumin, 45,000; and carbonic anhydrase, 29,000. A duplicate experiment gave similar results.

Table IV. Morphometry of Hamster Lungs 10 Wk after Intratracheal Instillation of Proteinases

Group	N	Lung morphometry			Average lung volume
		L_m	ISA	ISA ₄	
		μm	m^2	m^2	ml
Saline	10	49.7±0.7*	0.434±0.006	0.375±0.005	5.4±0.13
OP-P4	4	81.9±8.1†	0.237±0.026	0.213±0.024	4.7±0.24
PR-3	4	71.6±0.8†	0.254±0.004	0.223±0.004	5.1±0.11
HLE	6	66.9±5.6†	0.376±0.029	0.290±0.023	5.9±0.17

The lungs were excised, fixed in formalin at constant pressure (25 cm H₂O). Lung volumes were determined by water displacement. ISA was calculated from the lung volumes and alveolar intercepts and corrected to a constant lung volume of 4 ml (ISA₄).

* Values are expressed as mean±SE.

† Significantly different for L_m value for saline ($P < 0.05$).

PMSF and DFP), they found that PR-3 did not stain with the elastase-specific substrate, *N*-acetyl-D,L-alanine α -naphthyl ester, or the cathepsin G specific substrate, *N*-acetyl-D,L-phenylalanine β -naphthyl ester.

The characterization studies of purified PR-3 carried out in the current investigation fully confirmed and extended their results. By both SDS-PAGE and nondenaturing disk gel electrophoresis, a set of multiple bands were seen, indicating the existence of iso-forms of the slow-migrating PR-3. The molecular weight of the major band was 26,800 D, similar to HLE or cathepsin G. PR-3 exhibited maximal esterase activity at pH 7.0, indicating it is a neutral proteinase. The quantity purified was approximately one-third (by weight) of HLE. PR-3 was inhibited by PMSF or DFP, but not by metallo, thiol, or carboxyl proteinase inhibitors, identifying it as having a serine active site. It hydrolyzed elastin, hemoglobin, and was weakly active against azocasein. It was found to be inactive against HLE specific substrates, a cathepsin G-specific substrate or a trypsin specific substrate. The isoelectric point of PR-3 was substantially lower than that of HLE or cathepsin G indicating that it was less cationic. PR-3 was not precipitated by a polyclonal rabbit anti-HLE indicating that it was immunologically distinct from HLE. We also have preliminary experiments that demonstrate that PR-3 is contained in the azurophilic granules of PMN (not shown).

Although the precise relationship of PR-3 to HLE and cathepsin G will require further study, several implications can be derived from the current investigation. The failure of PR-3 to hydrolyze chromogenic peptides specific for HLE or cathepsin G and to be inhibited by peptide chloromethyl ketones specific for these enzymes, suggests qualitative differences in the manner in which PR-3, HLE, and cathepsin G degrade elastin. HLE degrades elastin by attacking peptide bonds on the carboxyl side of valine and to a lesser extent alanine (31). Cathepsin G attacks primarily bonds on the carboxyl side of tyrosine and phenylalanine (32). The preferred site of attack by PR-3 has not been identified. It is possible, although unlikely, that PR-3 did not show activity against the synthetic substrates because of their low molecular weight and not due to a lack of specificity toward the peptide residues that they contain. Studies such as those by Blow et al., which have used the oxidized B chain of insulin to map the preferred peptide bonds for HLE

(31) and cathepsin G (32) will be necessary to elucidate the specificity of PR-3. Collectively, however, the results with the chromogenic substrates and inhibitors suggest that the preferred site of attack of PR-3 is different from that of HLE or cathepsin G. Nevertheless, PR-3 is a true elastase. Its ability to degrade ligamentum nuchae elastin actually exceeded that of HLE (weight/weight) at pH 6.5.

The lower pI of PR-3 compared to HLE also has potentially important implications. Previous studies have shown that several anionic and cationic substances stimulate HLE-elastin interactions. The former appear to work by rendering elastin more anionic and thus facilitating binding of the cationic HLE (33). The cationic agents, which include platelet factor 4 (PF₄) and histones, probably act by occupying anionic, nonproductive sites and thus directing HLE activity to productive binding sites such as alanine or valine residues (34). Indeed, studies have shown that PF₄ can potentiate HLE-induced emphysema (35). While additional studies are needed, it is unlikely that PF₄ would have a similar influence on PR-3 because of its substantially lower pI. This lower pI probably accounts for its maximal activity at a neutral pH that is lower than the pH optimum for HLE activity. Thus regulation of the elastolytic action of HLE and PR-3 in vivo may be considerably different.

Analogous to HLE and cathepsin G, the results presented suggest that PR-3 contains at least three distinct isoenzymes. For HLE, these differences are felt to be due to differences in glycosylation (36). Further studies are needed to determine whether this is also the basis for the multiple forms of PR-3. A less likely possibility is that only a single PR-3 species exists, but that it is altered during the preparative procedures in such a way that artifact subspecies are produced with slightly differing electrophoretic mobilities.

We believe that the mechanism by which PR-3 produces emphysema relates directly to its ability to degrade elastin. Many connective tissue matrix abnormalities have been implicated in emphysema and the other PMN neutral serine proteinases have broad substrate specificity. HLE can degrade many connective tissue architectural components in vitro including the core protein of proteoglycans (37), type III and type IV collagens (38, 39), and fibronectin (40); cathepsin G can degrade proteoglycans (37), fibronectin (41), and laminin (42). If PR-3 is similar to HLE or cathepsin G in exhibiting broad substrate specificity, it is possible that matrix components other than elastin may be its primary targets. Indeed, we have preliminary evidence that PR-3 degrades fibronectin, type IV collagen and the core protein of proteoglycans. Since the procedures of elastin purification of necessity resort to relatively harsh extraction conditions to solubilize the non-elastin components, a degree of uncertainty exists regarding the susceptibility of elastin within a complex extracellular matrix to degradation by PR-3. However, studies to date have shown that there is a strong correlation between the emphysema producing potential of various enzyme preparations and their in vitro elastin degrading activity (43).

The major defense against lung damage by extracellular elastolytic enzymes probably lies in proteinase inhibitors. One approach for limiting the damage to the lung is to increase the antiproteinase levels in the lung by supplementing with suitable inhibitors. To achieve this goal considerable research effort has been directed toward the study of elastase inhibitors, both natural and synthetic. Several specific inhibitors of HLE

are currently available, their efficacy has been tested in experimental models of emphysema (44–46) and the prospect for their use in man is being actively discussed (47, 48). It will be of considerable practical importance to determine what proportion PR-3 contributes to the lung elastolytic burden under selected circumstances since this may profoundly effect the types of inhibitors that could be employed. Clearly there is much to be learned about the physical and functional properties of PR-3 and its role in the induction of acute or chronic pulmonary damage as well as other destructive diseases such as atherosclerosis or arthritis.

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